IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Holm et al.	Art Unit	: 1644
Serial No.	: 10/001,245	Examiner	: Nora Maureen Rooney
Filed	: November 15, 2001	Conf. No.	: 9286

Title : **NOVEL MUTANT ALLERGENS**

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF UNDER 37 C.F.R. 41.37

This is the Appeal Brief for the Notice of Appeal that was filed in the above-identified application on July 9, 2012. A petition for an extension-of-time under 37 C.F.R. 1.136(a) and the required fee are submitted herewith, to extend the time for filing the Appeal Brief to October 9, 2012.

No additional fee is believed to be due. The Commissioner is authorized to charge any additional fees determined to be due and requested to credit any overpayments to deposit account no. 06-1050.

Pursuant to the Rules of Practice Before the Board of Patent Appeals and Interferences in Ex Parte appeals, Final Rule 76 FR 72270 (Nov. 22, 2011), Applicants submit as follows:

(1) Real Party in Interest

The real party of interest is ALK-Abello A/S, Boge-Alle 6-8, DK2970 Horsholm, DK, by virtue of an assignment from the inventors recorded at reel/frame 013534/0232.

(2) Related Appeals and Interferences

None.

(3) Summary of Claimed Subject Matter

Claims 1-22, 35, 37-39 and 66-85 are pending. Claims 16 and 18-22 have been withdrawn from consideration by the Examiner as being directed to non-elected subject matter.

The claimed subject matter is directed to recombinant mutant allergens that are useful for allergy immunotherapy, with reduced risk of anaphylactic reactions. Specification at page 18, line 36 - page 19, line 3. The claimed recombinant mutant allergens derive these advantages by mutating surface-exposed amino acids that are likely to be part of different, spaced allergenic epitopes (i.e., IgE binding epitopes), while preserving a sufficiently large continuous surface on the recombinant mutant allergens, such that they preserve sufficient surface area to illicit antibodies that will bind to the naturally occurring allergen. *See* Specification at page 18, line 1-page 19, line 4 and page 20, lines 1-17. As a result, the claimed recombinant mutant allergens reduce allergen mediated cross-linking (and, hence, the possibility of an allergen-mediated response), while permitting the raising of an IgG response, wherein the IgG competes with pre-existing IgE. *Id.*

Claim 1 is the sole independent claim. As described in the specification and recited in claim 1, the claims are directed to a recombinant mutant allergen of a naturally occurring allergen (Specification at page 19, lines 21-23) selected from the group consisting of Fagales group 1 allergens (Specification at page 27, line 35; page 28, lines 19-22, reciting Fagales group 1 allergens, Bet v 1, Aln g 1 and Car b 1, page 43, lines 4-9, page 44, line 29 through page 45, line 5 and original claims 20 and 21), Vespidae antigen 5 allergens (Specification at Fig. 10, page 28, lines 15-16; page 28, lines 34-35, reciting Vespidae antigen 5 allergens Ves v 5 and Pol a 5, and original claims 32 and 33), house dust mite group 1 and group 2 allergens (Fig. 2, 5 and 32; page 28, lines 8-10 and 27-29) and grass group 5 allergens (Fig 38; page 27, line 34; page 28, lines 2-4; and page 28, lines 25-27, reciting grass group 5 allergens Lol p 5, Phl 5, Poa 5 and Sec 5). The recombinant mutant allergens comprise at least four mutations that are arranged such that they are spaced from each other by at least 15 Å and, further, such that they preserve at least one circular surface region area of 800 $Å^2$ on the mutant that comprises no mutation. Specification at page 19, lines 33-36. Each of the at least four mutations reduces the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of the naturally occurring allergen. Specification at page 19, lines 24-27. Each of the at least four mutations is a substitution of one surface-exposed amino acid residue with another residue that does not occur in the same position in the amino acid sequence of any

known homologous protein within the taxonomic species from which the naturally occurring allergen originates. Specification at page 19, lines 29-32.

(4) Argument

The pending rejections should be withdrawn for the following reasons:

(i) Obviousness-type Double Patenting Rejection

Claims 1-15, 35, 37-39 and 66-85 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting over certain claims of co-pending application no. 10/719,553 ("the '553 application"). The '553 application is under appeal. Applicants do not believe the pending claims are obvious variants of the claims of the '553 application. In order to reduce issues in the present appeal, however, Applicants state that if the instant rejection is the final remaining rejection in the application, Applicants will either file a request to expressly abandon the '553 application or submit a terminal disclaimer to overcome the rejection.

(ii) Rejections Under 35 U.S.C. §112, second paragraph (indefiniteness)

Introduction

Claims 1-15, 17, 35, 37-39 and 66-85 stand rejected for alleged indefiniteness. The Examiner's position is that the limitations of "being 'a mutant allergen of a naturally occurring allergen' and 'known homologous protein'" are unclear. The Examiner states that the scope of the claims would be changeable as more wild-type allergen sequences are identified. The rejection should be withdrawn because a person of ordinary skill in the art would not find the claimed subject matter indefinite.

The Standard for Indefiniteness

The standard for indefiniteness is whether "those skilled in the art would understand what is claimed when the claim is read in light of the specification." *Orthokinetics, Inc., v. Safety Travel Chairs, Inc.,* 806 F.2d 1565, 1576 (Fed. Cir. 1986). The claims are definite if "the claims, read in light of the specification, reasonably apprise those skilled in the art both of

the utilization and scope of the invention, and are as precise as the subject matter permits." *Hybritech, Inc., v. Monoclonal Antibodies, Inc.,* 802 F.2d 1367, 1385 (Fed. Cir. 1986).

The Examiner Has Failed to Meet the Offices' Burden of Showing the Claims Are Indefinite

The rejection should be withdrawn because the Examiner has failed to provide a supportable basis for concluding that one of ordinary skill in the art would not understand what is claimed when the claims are read in light of the specification. The claims call for a "recombinant mutant allergen of a naturally occurring allergen." The meaning of "naturally occurring allergen" is self-evident, i.e., an allergen that is obtained from the wild. A recombinant mutant allergen of a naturally occurring allergen is therefore simply a naturally-occurring allergen that has been mutated. Contrary to the Examiner's position, the phrase "recombinant mutant allergen of a naturally occurring allergen" is thus clear on its face. The phrase "known homologous protein" is similarly clear on its face.

The Examiner's position concerning the possibility that the scope of the claims may change as more wild-type allergens are identified is not an adequate basis for a rejection under § 112. Compliance with section 112 is measured against the knowledge of skill in the art at the time the application was filed. *See,* e.g., *Chiron Corp. v. Genentech*, Inc., 363 F.3d 1247, 1254 (Fed. Cir. 2004) (A "patent disclosure need not enable information within the knowledge of an ordinarily skilled artisan. . . . At the other end of the knowledge continuum, a patent document cannot enable technology that arises after the date of application. The law does not expect an applicant to disclose knowledge invented or developed after the filing date. Such disclosure would be impossible.") The possibility of the discovery of new allergens does not mean the claims are indefinite.

Lastly, the response to the Examiner's query concerning whether "naturally occurring sequences" comprising particular amino acids at positions listed on page 31, line 25 to page 32, line 2 of the specification is straight forward. The claims are directed to "recombinant mutant allergen[s] of a naturally occurring allergen". Naturally occurring allergens are not

"recombinant" allergens. Nor are they "mutant" allergens. Naturally occurring allergens in any form are thus not encompassed by the instant claims.

For reasons set forth above the claims "reasonably apprise those skilled in the art both of the utilization and scope of the invention, and are as precise as the subject matter permits." The claims are therefore not indefinite. The rejection under 35 U.S.C. §112, second paragraph should therefore be withdrawn.

(iii) Rejection Under 35 U.S.C. §112, first paragraph (written description)

Introduction

Claims 1-15, 17, 35, 37-39 and 66-85 stand rejected for alleged failure to comply with the written description requirement. The rejection should be withdrawn because the specification discloses sufficient information to show that the inventors were in possession of the full scope of the claimed invention when the application was filed and, moreover, do not overreach the scope of the inventors' contribution to the art. Compliance with the written description requirement requires no more. The rejection should therefore be withdrawn.

Overview of the Claimed Invention

The advantages of the claimed recombinant mutant allergens are that they exhibit reduced allergenicity, compared to the naturally occurring allergens from which they are derived, yet retain the immunogenicity (i.e., the ability to induce an immune response) required to illicit a protective immune response. The rejected claims are directed to recombinant mutant allergens of a naturally occurring allergen selected from the group consisting of Fagales group 1 allergens, Vespidae antigen 5 allergens, house dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens. *See* claim 1. The claims are thus directed to recombinant mutant allergens derived from Fagales group 1 allergens, Vespidae antigen 5 allergens, house dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens and comprising at least four mutations, which each reduce the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of the naturally occurring allergen, each of said at least four mutations being a substitution of one surface-exposed amino acid

residue with another residue, which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which said naturally occurring allergen originates, each of said at least four mutations being spaced from each other by at least 15 Å, and comprising at least one circular surface region with an area of 800 $Å^2$ that comprises no mutation.

The Written Description Standard

The specification provides adequate written description for the claimed recombinant mutant allergens. The written description requirement requires that the specification provide disclosure that allows one of ordinary skill in the art of the invention to "recognize that [the inventor] invented what is claimed." *Lockwood v. American Airlines, Inc.,* 107 F.3d 1565, 1572 (Fed. Cir. 1997); *see also Vas-Cath Inc. v. Mahurkar,* 935 F.2d 1555, 1563-64 (Fed. Cir. 1991) (Applicant "must convey with reasonable clarity to those skilled in the art that ... he or she was in possession *of the invention*.") (emphasis in original). The written description requirement "ensure[s] that the scope of the right to exclude, as set forth in the claims, does not overreach the scope of the inventor's contribution to the field of art as detailed in the patent specification." *Reiffin v. Microsoft Corp.,* 214 F.3d 1342, 1354 (Fed. Cir. 2000); *Amgen Inc. v. Hoechst Marion Roussel, Inc.,* 314 F.3d 1313, 1330 (Fed. Cir. 2003) ("The purpose of the written description requirement is to prevent an applicant from later asserting that he invented that which he did not."). When claiming a genus, the written description requirement is met by providing sufficient structural, physical and/or functional properties that describe the genus and/or describing sufficient members of the genus such that it shows the inventors were in possession of the genus. *Regents of the University of California v. Eli Lilly & Co.,* 119 F.3d 1559, 1567-68 (Fed. Cir. 1997). Functional language may provide adequate written description "if in the knowledge of the art the disclosed function is sufficiently correlated with a particular, known structure." *Amgen Inc. v. Hoechst Marion Roussel, Inc.,* 314 F.3d at 1332 (Fed. Cir. 2003) *citing Enzo Biochem, Inc. v. Gen-Probe, Inc.,* 296 F.3d 1316, 1324 (Fed. Cir. 2002).

The Specification Sets Forth the Claimed Invention in Sufficient Detail to Show Applicants were in Possession of the Claimed Invention

The specification discloses that "the invention is based on the recognition that a mutated allergen having IgE binding reducing mutations in multiple B-cell epitopes, and at least one intact epitope" would reduce crosslinking IgE, and thus the allergenicity of the mutant allergens, while preserving at least one epitope to raise an IgG response. Specification at page 18, lines 29-36. The specification discloses that the recombinant mutant allergens are produced by making substitutions of at least four surfaced-exposed, conserved amino acids that are spaced from each other by at least 15 Å, while preserving at least one circular surface region of 800 $Å^2$. Specification at, e.g., page 19, line 21-page 20, line 1. The spacing of the at least four mutations ensures that they are in separate clusters of epitopes. Specification at page 20, lines 14-17. In addition to the at least four mutations spaced at least 15 Å from each other, the recombinant mutant allergens may further comprise additional mutations ("secondary mutations") that further reduce IgE binding. Specification at page 24, line 27 through page 25, line 8. These additional mutations are also placed in such a manner so as to preserve at least one circular surface region of 800 $Å^2$ with no mutation. Specification at page 25, lines 2-3. The specification further sets forth detailed "Criteria for substitution." Specification at page 36-38.

The specification further gives detailed analysis on the structural features of Bet v 1, Der p 2, Ves v 5, Der p 1, Phl p 5 and related proteins that further show possession of the full scope of the claimed invention. The specification discloses 57 amino acids of Bet v 1 that are highly solvent exposed and conserved (page 68), 54 amino acids of Der p 2 that are highly solvent exposed and conserved (page 72), 88 amino acids of Ves v 5 that are highly solvent exposed and conserved (page 76) and sets forth 12 Der p 2 mutants (pages 97-98), 11 Der p 1 mutants (pages 105-106), 14 Phl p 5 mutants (pages 114-115). The detailed description of amino acids to be mutated and the combinations of mutants demonstrate that the inventors had possession of the claimed invention as it relates to Bet v 1, Ves v 5, Der p 1, Der p 2, and Phl p 5. Moreover, as disclosed in the specification, Bet v 1, Ves v 5, Der p 1, Der p 2, and Phl p 5 are highly homologous to allergens Fagales group 1 allergens, Vespidae antigen 5 allergens, house

dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens, respectively. See specification at page 81, lines 1-15 (67 sequences homologous to Bet v 1 within the order Fagales), page 58 and Fig. 10 A (Vespula Antigen 5s about 90% identical), Fig. 35 A and B (sequence alignment of Der p 1 and other house dust mite group 1 allergens), Fig. 32 (sequence of Der p 2 with other house dust mite group 2 allergens), and Fig. 38 A-D (sequence alignment of Phl p 5 with other grass group 5 allergens). One of ordinary skill in the art would understand that the high degree of sequence identity among the members of the respective allergen families recited in the claims means that description of recombinant mutant allergens for a single member of the family provides written description for recombinant mutant allergens of any allergen within the same family. Thus, the specification provides written description for the recombinant mutant allergens called for in the claims.

Moreover, at the time the application was filed, the state of the art in the field of the invention was high. At the time the invention was made, many of the allergens set forth in the specification had been cloned or purified and sequenced, the three dimensional structure of many of the allergens determined, and multiple homologues of many of the allergens had been sequenced, allowing for comparison among family members. The specification discloses, for example, that three dimensional structures had been published for Bet v 1, Ves v 5 and Der p 2 (specification at page 67, lines 7-8 (3-D structure of Bet v 1 available from Protein Data Bank (PDB) - PDB identifier 1bv1), page 67, lines 23-24 (3-D structure of Der p 2 available as PDB identifier 1bv1), page 67, line 36-page 68, line 1 (3-D structure of Ves v 5 based on accession number Q05110)). Although the specification sets forth that the Ves v 5 coordinates are unpublished, the PDB entry states that the Ves v 5 structure was released on October 26, 2000. *See* Tab A, submitted with response dated October 31, 2007. Additionally, at the time the application was filed, a robust 3-D structure of Der p 1 had been published (Topham et al., 1994, Protein Eng. 7:869-894, see Tab B, submitted with response dated October 31, 2007). Additionally, the specification sets forth molecular models for Bet v 1 (Fig. 1-26 and 30), Der p 2 (Fig. 33 and 34), Der p 1 (Fig. 36 and 37) and Phl p 5 (Fig. 39 and 40).

The structures of additional allergens were also known when the application was filed. King et al., U.S. Patent Publication No. 2003/0039660 A1 ("King"), which is cited in the specification, for example, lists allergens in the protein database for which the three dimensional structure was known. *See* King at Table 8, beginning at page 51, listing references for allergens that have been cloned and/or sequenced and Table 9, beginning at page 67, listing allergens in the protein database for which the three dimensional structure was known. It is noted that although King has a filing date of March 3, 2002, the vast majority of the references cited in Tables 8 and 9 have publication dates and/or deposit dates that are prior to the earliest claimed priority date of the instant application. Applicants' study of the protein database indicates that at the time the application was filed, experimentally determined structures were available at least for Api m 1, Bet v 1, Bet v 2 (birch pollen profiling I), Phl p 2, Ara t 8 (Arabidopsis thaliana profilin I), Acanthamoeba castelllani profiling IA, Der f 2, Amb t 5 (Amb V), Der p 2, Bos d 2, Equ C 1, Acanthamoeba castellanii profilin II, Api m 2 and Ves v 5.

Furthermore, to the extent they might be required, at the time the application was filed, it would have been routine to determine additional 3-D structures for allergens. Abola et al., *Nat. Struct. Biol.*, November 2000, 7(Suppl):973-7 (Attached at Tab A), provides evidence that when the application was filed crystallographic methods were so far advanced, and had become a matter of routine to such extent, that they were ripe for automation. The field had thus advanced to the point where it was routine to determine the structure of individual proteins. Robotic systems that were capable of performing up to "> 100,000" crystallization trials a day already existed (Abola at page 973, col. 2, para. 2). With such systems available, the optimization of crystallization conditions to obtain crystals suitable for structure determination, had become accessible to a person of ordinary skill in the art of structural biology.

Thus, as of the filing date of the application, one of ordinary skill in the art would immediately recognize that the listing of allergens set forth in the specification referred to amino acid sequences of allergens that were known and that such amino acid sequences were readily ascertainable.

With respect to the functional limitation of reducing IgE binding, the specification teaches that the mutations called for in the claims are directed to dominant IgE epitopes. The specification further teaches that the reduction in IgE binding called for in the claims may be determined using any immunoassay known in the art or by assessing reduced IgE binding and the reduced ability of a mutant to initiate histamine release. *See*, e.g., specification at page 26, lines 11-30.

Lastly, the specification exemplifies the claimed invention with a dozen mutants of the birch allergen Bet v 1 (specification at, e.g., page 29, line 19 through page 30, line 31 and page 97, line 35 through page 98, line 34), eleven mutants of the dust mite allergen, Der p 1 (specification at page 105, line 10 through page 111, line 25), and 14 mutations of the grass allergen, Phl p 5 (specification at page 111, line 28 through page 120, line 26). The specification thus provides 37 examples from three completely unrelated allergens that fall within the scope of the claimed invention.

In short, the specification teaches that the starting materials for the claimed recombinant mutant allergens are known proteins, i.e., naturally occurring allergens, and further gives clear teachings on how to use such starting materials to arrive at the claimed invention. The state of the art, moreover, is high. One of ordinary skill in the art would thus immediately envision that the claimed recombinant mutant allergens bearing the mutations made according the teachings of the specification would largely retain the structure and antigenicity of wild-type allergens from which they are derived. The Examiner has failed to adduce sufficient evidence to the contrary.

The Written Description Case Law Cited by the Examiner Does NOT Lead to a Conclusion that the Claims Lack Written Description

In setting forth the instant rejection, the Examiner has cited *Eli Lilly*, *supra*. The nature of the instant invention and the disclosure of the instant specification, however, are very different from *Eli Lilly*. In *Eli Lilly*, the Federal Circuit held that the disclosure of the sequence of a rat insulin cDNA did not provide adequate written description for the insulin cDNA

sequences of every vertebrate. *Eli Lilly* at 1566-67. In *Eli Lilly*, however, the specification failed to provide any features that described the claimed vertebrate insulin cDNA. The Court found that the claimed cDNAs were described solely by their function or how to obtain them. The instant case is inapposite to *Eli Lilly*. In *Eli Lilly* the claims were directed to unknown cDNA sequences. The instant claims, by contrast, are drawn to mutant allergens that are derived by making substitutions in a family of allergens, i.e., Fagales group 1 allergens, Vespidae antigen 5 allergens, house dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens, with closely related sequences. In *Eli Lilly*, no structural features were provided that correlated with the function of the claimed vertebrate insulin cDNA. In the instant case, the specification provides that substituted amino acids are those amino acids that are conserved, solvent accessible amino acids that are spaced at least 15 Å from each other and which are each outside a circular area of 800 $Å^2$ on the surface of the allergen and goes on to list particular amino acids to choose among to make the claimed recombinant mutant allergens.

Nor does the decision of the Board of Patent Appeals and Interferences in *ex parte Kubin* (83 USPQ2d 1410 (BPAI 2007[1])) support a finding that the instant specification fails to provide adequate written description for the pending claims. In *Kubin*, the Board upheld the rejection of a claim directed to isolated polynucleotides encoding polypeptides that (1) "are at least 80% identical to amino acids 22-221 of SEQ ID NO: 2" (i.e., the amino acid sequence for the extracellular domain of the protein natural killer cell activation inducing ligand ("NAIL") lacking the NAIL signal sequence) and (2) which bind to the glycoprotein CD 48. *Id.* at 1417. The specification in *Kubin* disclosed the sequence of two nucleic acids within the scope of the claim and three fusion proteins whose nucleic acid sequences would fall within the scope of the claim. *Id.* None of these sequences varied amino acids 22-221 of SEQ ID NO: 2. *Id.*

The Board in *Kubin* found that the Applicant had failed to describe what domains within amino acids 22-221 of SEQ ID NO: 2 correlated with the function of binding CD 48, and

[1] The Court of Appeals for the Federal Circuit affirmed the Board's decision in Kubin on the grounds that the claims were obvious over the prior art. *In re Kubin,* 561 F.3d 1351, 1358-61 (Fed. Cir. 2009). The Federal Circuit, however, declined to address the question of whether the claims in Kubin were properly rejected for failure to comply with the written description requirement. *Id.* at 1361.

thus the Applicant had not described which NAIL amino acids could be varied and still maintain CD 48 binding. *Id.* Citing *Eli Lilly*, the Board found that in the absence of a structure-function correlation, the claim merely defined the invention by function, which was not sufficient to satisfy the written description requirement.

Kubin is distinguished from the instant case for much the same reasons as *Eli Lilly*. In *Kubin*, the Applicant failed to provide any features of amino acids 22-221 of SEQ ID NO: 2 that correlated with binding to CD 48. As set forth above, the instant specification, in contrast, allows one of ordinary skill in the art to identify amino acids of Fagales group 1 allergens, Vespidae antigen 5 allergens, house dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens. Furthermore, whereas in *Kubin* the Applicant failed to disclose any polynucleotides encoding NAIL protein that varied in amino acids 22-221, the instant application identifies numerous amino acids for substitution in Fagales group 1 allergens, Vespidae antigen 5 allergens, house dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens, and further sets forth examples of combinations of mutants, whereas the Applicant in *Kubin* failed to provide any working examples of polynucleotides encoding a polypeptide at least 80% identical to amino acids 22-221 of SEQ ID NO: 2 and which bind CD 48.

In short, as with *Eli Lilly*, the Applicant in *Kubin* failed to provide any structural features that correlated with the function of the polypeptide called for in the claim, whereas the instant specification sets out the features, including specific amino acids, of Fagales group 1 allergens, Vespidae antigen 5 allergens, house dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens that are called for in the claims and which allow one of ordinary skill in the mutant art to make the claimed recombinant allergens. Thus, the basis of the Board's decision in *Kubin* does not apply to the instant claims.

The Examiner Fails to Provide a Rationale as to Why the 37 Recombinant Mutant Allergens Exemplifed in the Specification Are Not Sufficient to Show the Inventors had Possession of the Full Scope of the Claimed Invention

Applicants may show possession of a claimed genus by describing "sufficient members" *Regents of the University of California v. Eli Lilly & Co.,* 119 F.3d at 1567-68. Here, the specification exemplifies the claimed invention with a dozen mutants of the birch allergen Bet v 1, eleven mutants of the dust mite allergen, Der p 1, and 14 mutations of the grass allergen, Phl p 5. The examples thus cover 37 members of the claimed invention from three completely unrelated allergens. The Examiner has failed to provide a legitimate rationale as to why the number and diversity of exemplified species is not sufficient to show the inventors were in possession of the full scope of the claimed invention.

The Examiner Applies an Overly Stringent Standard for Written Description and Conflates the Written Description Requirement with Other Requirements Set Forth in Section 112

The specification teaches that the starting point for the genus of recombinant mutant allergens are known allergens and that the claimed recombinant mutant allergens are made as described. The features of each recombinant mutant allergen, moreover, are set forth in the claims. One of ordinary skill in the art would immediately recognize that the recombinant mutant allergens that retain their three-dimensional would largely retain the structure of the naturally occurring allergens from which they were derived. The specification thus includes written descriptive support for the claimed mutant allergens.

The structure of Bet v 1 was known at the time the application was filed and Bet v 1 allergens are highly conserved. There is no rule that the Applicants provide description of the precise mutant amino acids in the claimed recombinant Bet v 1 mutants. *Falkner v. Inglis,* 448 F.3d 1357, 1366 (Fed. Cir. 2006). Applicants are entitled to "flexibility" in how they claim their invention. *Univ. of Rochester v. G.D. Searle & Co., Inc.,* 358 F.3d 916, 927-928 (Fed. Cir. 2004). In *Ariad v. Eli Lilly*, the Federal Circuit reiterated, "[written description] doctrine never created a heightened requirement to provide a nucleotide-by-nucleotide recitation of the entire genus of claimed genetic material; it has always expressly permitted the disclosure of structural features common to the members of the genus." *Ariad Pharmaceuticals, Inc. v. Eli Lilly and Co.,* cv 2008-1248, Fed. Cir., *en banc,* decided March 22, 2010, slip op at 26, *citations omitted.*

Here, when measured against the known, conserved structure of Bet v 1 allergens and the high level of skill in the art concerning B-cell epitopes, the claims tell one of ordinary skill in the art where mutations are placed in the claimed recombinant allergens. The claims thus describe the claimed invention and do not "merely [draw] a fence around the outer limits of a purported genus." *Id.* at 21.

Applicants are not claiming the discovery of any allergen. As discussed above, the naturally occurring allergens that are the starting point for the claimed invention were known in the art at the time the application was filed. The 3-D structures of the naturally occurring allergens were also known, or were readily ascertainable. Thus, here, as in Capon, the "invention does not concern the discovery of gene function or structure, as in Lilly." *Capon v. Eshhar,* 418 F.3d 1349, 1358 (Fed. Cir. 2005). (Claiming genes "produced by selecting and combining known heavy- and light-chain immune-related DNA segments, using known DNA-linking procedures." *Id.* at 1355.) The instant rejection implies that the written description requirement requires that the inventors describe every permutation of recombinant mutant allergen for every allergen. This is not the standard for compliance with the written description requirement. As the court held in Capon, "[i]t is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention." *Id.* at 1359. In the instant case, the specification describes the claimed invention in terms of mutations set out in characterized proteins, i.e., allergens, in such a manner as to affect a well characterized property of the proteins, i.e., allergenicity. The Examiner has failed to establish why this information is insufficient to demonstrate the inventors were in possession of a generic invention.

The Examiner also raises the point that the claims cover many different recombinant mutant allergens. The Examiner, however, has failed to provide a rationale that supports a finding that a structural description of additional allergens would have been required in order for a person of ordinary skill in the art to recognize Applicants were in possession of the claimed genus of recombinant mutant allergens. *See* Ariad Pharms., Inc., v. Eli Lilly & Co., 598

F.3d 1336, 1351 (Fed. Cir. 2010) (en banc) ("[T]he test for sufficiency is whether the disclosure of the application relied upon reasonably conveys to those skilled in the art that the inventor had possession of the claimed subject matter as of the filing date.")

Throughout the course of prosecution, the Examiner has repeatedly copied extensive text from the claims language and Applicants' arguments. *See*, e.g., pending Office Action at pages 6 through 15). The Examiner also expends considerable space discussing the Bet v 1 superfamily. See pending Office Action at page 15 through 18. The Examiner, however, fails to provide a cogent rationale as to how this extended verbiage shows why a person of ordinary skill in the art would require more than the information disclosed in the specification to recognize that Applicants were in possession of the generic invention that is defined by the claims. As set out by the Federal Circuit in Ariad, "[t]he test for sufficiency is whether the disclosure of the application relied upon reasonably conveys to those skilled in the art that the inventor had possession of the claimed subject matter as of the filing date." Ariad at 1351.

The Examiner thus states that "[t]he written description requirement is to adequately identify what one has invented to prevent an applicant for patent from perpetrating a fraud on the public and later claiming exclusive rights to that what he or she did not invent." Office Action at page 18. The Examiner fails to show how this applies to the instant invention. To the contrary, as discussed above, the specification describes full scope of the claimed invention. The implication that the grant of the instant claims would amount to "perpetrating a fraud on the public" is overreaching on the part of the Examiner.

The Examiner further states, "The patentee must disclose in the patent sufficient information to put the public in possession of the invention and to enable those skilled in the art to make and use the invention." *Id.* This statement conflates the written description and enablement requirements. Moreover, with respect to enablement, the specification enables the full scope of the claimed invention. In fact, the Examiner originally rejected the claims for alleged lack of enablement, and then withdrew the rejection based upon Applicants' arguments.

The Examiner, further states, "The sufficiency of written description requirement is important for those of ordinary skill in the art to know what is being claimed so as not to

infringe the patented claims and after the patent expires to be able to know what is then in the public realm." Office Action at page 18. Here the Examiner conflates the written description requirement and section 112, second paragraph. Moreover, as set forth above, the claims comply with the requirement for definiteness set forth in section 112, second paragraph.

When viewed against the proper standard, it is evident that the inventors have met the written description for protein-based invention. The specification is thus NOT a "description of what a material does, rather than what it is." *Rochester,* 358 F.3d at 923; *Eli Lilly,* 119 F.3d at 1568. Nor does the invention "merely [describe] how to obtain possession of members of the claimed genus." *Ex parte Kubin,* 83 USPQ3d at 1417, *citing Rochester,* 358 F.3d at 927. To the contrary, as set out above, the specification lays out the claimed invention in precise terms that readily demonstrate possession of the claimed invention. "The burden of showing that the claimed invention is not described in the application rests on the PTO in the first instance." *In re Edwards,* 568 F.2d 1349, 1354 (CCPA 1978). The Examiner has not met this burden.

Additionally, the Examiner's statements that "Applicants have no way of knowing how to modify as yet undiscovered allergens that may differ from known allergens in ways that cannot be contemplated" and there "is no way to know what is or is not an allergen encompassed by the scope of the claims given the information disclosed in the specification" are not well taken. First the possibility that, notwithstanding the conserved structure of antigenicity among allergens in the same family, "undiscovered allergens that may differ from known allergens in ways that cannot be contemplated" is pure conjecture on the basis of the Examiner is therefore of little probative value. Contrary to the Examiner's assertion, the specification teaches precisely how to modify as yet undiscovered naturally-occurring allergens to arrive at the claimed invention. Lastly, the Examiner's statements concerning the scope of the claims blur the requirements for indefiniteness, enablement, and written description. These are additional reasons why the written description rejection should be withdrawn.

Lastly, the Examiner's comments concerning the references Radauer et al. and Smith et al. are not well taken. The Examiner goes on at length about purported phylogenetic relationships among Bet v 1-related sequences and sequence alignments among allergens from

different species. The instant claims, however, call for substitution of surface-exposed amino acid residues with another amino acid residue that does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which the naturally occurring allergen originates. *See* claim 1. The Examiner has failed to measure the content of Radaurer et al. and Smith against the claimed invention.

Conclusion

The specification provides sufficient written description to show Applicants were in possession of the full scope of the claimed invention when the application was filed. The Examiner has failed to adduce evidence as to why the specification fails to show the Applicants were in possession of the claimed invention when the application was filed. The Examiner has also applied an overly stringent standard for written description and conflated the written description requirement for other requirements set forth in section 112. For at least these reasons, the written description rejection should be withdrawn.

Conclusion

The application is believed to be in condition for allowance. The Board is requested to withdraw all rejections and instruct the Examiner to allow the application.

Respectfully submitted,

Date: October 9, 2012

/Mitchell Bernstein, Reg. No. 46,550/
Mitchell Bernstein
Reg. No. 46,550

Customer Number 26211
Fish & Richardson P.C.
Telephone: (212) 765-5070
Facsimile: (877) 769-7945

Appendix of Claims

1. (Previously presented) A recombinant mutant allergen of a naturally occurring allergen,

said naturally occurring allergen selected from the group consisting of Fagales group 1 allergens, Vespidae antigen 5 allergens, house dust mite group 1 allergens, house dust mite group 2 allergens and grass group 5 allergens and comprising at least four mutations, which each reduce the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of said naturally occurring allergen,

each of said at least four mutations being a substitution of one surface-exposed amino acid residue with another residue, which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which said naturally occurring allergen originates,

each of said at least four mutations being spaced from each other by at least 15 Å, and

said mutant allergen comprising at least one circular surface region with a area of 800 $Å^2$ that comprises no mutation.

2. (Previously presented) A recombinant mutant allergen according to claim 1, wherein the each of said at least four mutations is spaced from each other by between about 20 to 30 Å.

3. (Previously presented) A recombinant mutant allergen according to claim 1 which comprises at least five mutations in total, which each reduces the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of said naturally occurring allergen,

each of said at least five mutations in total being a substitution of one surface-exposed amino acid residue with another residue, which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which said naturally occurring allergen originates, and

at least two of said at least five mutations in total being spaced within 15 Å of each other.

4. (Previously presented) A recombinant mutant allergen according to claim 1, wherein at least one of the surface-exposed amino acids to be substituted in the naturally occurring allergen has a solvent accessibility of above 20 %.

5. (Previously presented) A recombinant mutant allergen according to claim 1, wherein at least one of the surface-exposed amino acids to be substituted in the naturally occurring allergen is conserved with more than 70 % identity in all known homologous proteins within the species from which said naturally occurring allergen originates.

6. (Previously presented) A recombinant mutant allergen according to claim 1, which essentially has the same α-carbon backbone tertiary structure as said naturally occurring allergen.

7. (Previously presented) A recombinant mutant allergen according to claim 1, wherein each amino acid residue to be incorporated into the mutant allergen does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic genus from which said naturally occurring allergen originates.

8. (Previously presented) A recombinant mutant allergen according claim 1, characterised in that the specific IgE binding to the mutated allergen is reduced by at least 5%.

9. (Previously presented) A recombinant mutant allergen according to claim 6, characterised in that when comparing the α-carbon backbone tertiary structures of the mutant and the naturally occurring allergen molecules, the average root mean square deviation of the atomic coordinates is below 2Å.

10. (Previously presented) A recombinant mutant allergen according to claim 1, characterised in said circular surface region comprises atoms of 15-25 amino acid residues.

11. (Previously presented) A recombinant mutant allergen according to claim 1, characterised in that the surface-exposed amino acid residues are ranked with respect to solvent accessibility, and that one or more amino acids among the more solvent accessible ones are substituted.

12. (Previously presented) A recombinant mutant allergen according to claim 1, characterised in that the surface-exposed amino acid residues are ranked with respect to degree of conservation in all known homologous proteins within the species from which said naturally occurring allergen originates, and that one or more amino acids among the more conserved ones are substituted.

13. (Previously presented) A recombinant mutant allergen according to claim 1, wherein the mutant allergen is a non-naturally occurring allergen.

14. (Previously presented) A recombinant mutant allergen according to claim 1 comprising from 5 to 20 mutations that reduce the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of said naturally occurring allergen,

each of said 5 to 20 mutations being a substitution of one surface-exposed amino acid residue with another residue, which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which said naturally occurring allergen originates, and

each of said 5 to 20 mutations being spaced from each other by at least 15 Å.

15. (Previously presented) A recombinant mutant allergen according to claim 3, which comprises at least 8 total mutations and wherein each of said at least four mutations spaced from each other by at least 15 Å is spaced within 15 Å of 1 to 4 of said at least 8 total mutations.

16. (Withdrawn/Previously presented) A recombinant mutant allergen according to claim 1 wherein said naturally occurring allergen is a grass group 5 allergen selected from the group consisting of Lol p 5, Phl p 5, Poa p 5 and Sec c 5.

17. (Previously presented) A recombinant mutant allergen according to claim 1 wherein said naturally occurring allergen is a house dust mite group 2 allergen selected from the group consisting of Der p 2, Der f 2 and Lep d 2.

18. (Withdrawn/Previously presented) A recombinant mutant allergen according to claim 1 wherein said naturally occurring allergen is a Fagales group I allergen selected from the group consisting of Bet v 1, Aln g 1, Cor a 1 and Car b 1.

19. (Withdrawn/Previously presented) A recombinant mutant allergen according to claim 1 wherein said naturally occurring allergen is a Vespidae antigen 5 allergen selected from the group consisting of Ves v 5 and Pol a 5.

20. (Withdrawn/Previously presented) A recombinant mutant allergen according to claim 1 wherein said naturally occurring allergen is a house dust mite group 1 allergen selected from the group consisting of Der p 1, Derf f 1 and Lep d 1.

21. (Withdrawn/Previously presented) A recombinant mutant allergen according to claim 18, characterised in that it is a mutant of Bet v 1.

22. (Withdrawn/Previously presented) A recombinant mutant allergen according to claim 21, characterised in that one or more of the substitutions is selected from the group consisting of V-2, D-72, E-87, K-129, E-60, N-7, K-65, P-108, N-159, D-93, K-123, K-32, D-125, R-145, D-109, E-127, Q-36, E-131, L-152, E-6, E-96, D-156, P-63, H-76, E-8, K-134, E-45, T-10, V-12, K-20, S-155, H-126, P-50, N-78, K-119, V-2, L-24, E-42, N-4, A-153, I-44, E-138, G-61, A-130, R-70, N-28, P-35, S-149, K-103, Y-150, H-154, N-43, A-106, K-115, P-14, Y-5, K-137, E-141, E-87 and E-73.

23-34. (Cancelled)

35. (Previously presented) A pharmaceutical composition comprising the recombinant mutant allergen according to claim 1 and at least one of a pharmaceutically acceptable carrier, excipient, or adjuvant.

36. (Cancelled)

37. (Previously presented) A composition comprising two or more recombinant mutant allergens according to claim 1, wherein each of said two or more recombinant mutant allergens respectively comprises at least one-mutation among said at least four mutations spaced at least 15 Å from each other that is at least 15 Å from any other mutation that is absent in at least one other of said two or more recombinant mutant allergens.

38. (Previously presented) A composition according to claim 37 comprising 2-12 recombinant mutant allergens.

39. (Previously presented) A composition according to claim 37 further comprising at least one of a pharmaceutically acceptable carrier, excipient, or adjuvant.

40-65. (Cancelled)

66. (Previously presented) The recombinant mutant allergen of claim 2 wherein said at least four mutations are spaced from each other by at least 25 Å.

67. (Previously presented) The recombinant mutant allergen of claim 1 wherein said at least four mutations are spaced from each other by at least 30 Å.

68. (Previously presented) The recombinant mutant allergen according to claim 4, wherein at least one of the surface-exposed amino acids to be substituted in the naturally occurring allergen has a solvent accessibility of above 30 %.

69. (Previously presented) The recombinant mutant allergen according to claim 68, wherein at least one of the surface-exposed amino acids to be substituted in the naturally occurring allergen has a solvent accessibility of above 40 %.

70. (Previously presented) The recombinant mutant allergen according to claim 69, wherein at least one of the surface-exposed amino acids to be substituted in the naturally occurring allergen has a solvent accessibility of above 50 %.

71. (Previously presented) A recombinant mutant allergen according to claim 5, wherein at least one of the surface-exposed amino acids to be substituted in the naturally occurring allergen is conserved with more than 80 % identity in all known homologous proteins within the species from which said naturally occurring allergen originates.

72. (Previously presented) A recombinant mutant allergen according to claim 71, wherein at least one of the surface-exposed amino acids to be substituted in the naturally occurring allergen is conserved with more than 90 % identity in all known homologous proteins within the species from which said naturally occurring allergen originates.

73. (Previously presented) A recombinant mutant allergen according to claim 7, wherein each amino acid residue to be incorporated into the mutant allergen does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic subfamily from which said naturally occurring allergen originates.

74. (Previously presented) A recombinant mutant allergen according to claim 73, wherein each amino acid residue to be incorporated into the mutant allergen does not occur in the

same position in the amino acid sequence of any known homologous protein within the taxonomic family from which said naturally occurring allergen originates.

75. (Previously presented) A recombinant mutant allergen according to claim 74, wherein each amino acid residue to be incorporated into the mutant allergen does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic superfamily from which said naturally occurring allergen originates.

76. (Previously presented) A recombinant mutant allergen according to claim 75, wherein each amino acid residue to be incorporated into the mutant allergen does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic legion from which said naturally occurring allergen originates.

77. (Previously presented) A recombinant mutant allergen according to claim 76, wherein each amino acid residue to be incorporated into the mutant allergen does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic suborder from which said naturally occurring allergen originates.

78. (Previously presented) A recombinant mutant allergen according to claim 77, wherein each amino acid residue to be incorporated into the mutant allergen does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic order from which said naturally occurring allergen originates.

79. (Previously presented) A recombinant mutant allergen according claim 8, characterised in that the specific IgE binding to the mutated allergen is reduced by at least 10%.

80. (Previously presented) A recombinant mutant allergen according to claim 14 comprising from 6 to 15 mutations that reduce the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of the said naturally occurring allergen,

each of said 6 to 15 mutations being a substitution of one surface-exposed amino acid residue with another residue, which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which said naturally occurring allergen originates, and

each of said 6 to 15 mutations being spaced from each other by at least 15 Å.

81. (Previously presented) A recombinant mutant allergen according to claim 80 comprising from 7 to 12 mutations that reduce the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of the said naturally occurring allergen,

each of said 7 to 12 mutations being a substitution of one surface-exposed amino acid residue with another residue, which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which said naturally occurring allergen originates, and

each of said 7 to 12 mutations being spaced from each other by at least 15 Å.

82. (Previously presented) A recombinant mutant allergen according to claim 81 comprising from 8 to 10 mutations that reduce the specific IgE binding capability of the mutated allergen as compared to the IgE binding capability of the said naturally occurring allergen,

each of said at said 8 to 10 mutations being a substitution of one surface-exposed amino acid residue with another residue, which does not occur in the same position in the amino acid sequence of any known homologous protein within the taxonomic species from which said naturally occurring allergen originates, and

each of said 8 to 10 mutations being spaced from each other by at least 15 Å.

83. (Previously presented) A composition according to claim 38 comprising 3-10 recombinant mutant allergens.

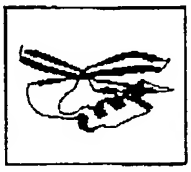
84. (Previously presented) A composition according to claim 83 comprising 4-8 recombinant mutant allergens.

85. (Previously presented) A composition according to claim 84 comprising 5-7 recombinant mutant allergens.

Automation of X-ray crystallography

Enrique Abola[1,2], Peter Kuhn[3], Thomas Earnest[4] and Raymond C. Stevens[1,2,5]

Structure-based biological discovery is entering a new era with the development of industrialized macromolecular structure determination pipelines. Intense, highly focused X-rays from integrated synchrotron radiation beam lines combined with significant advances in protein expression, purification, and micro-crystallization automation allow for the full streamlining of the traditionally tedious and time consuming process of determining the three dimensional structures of macromolecules.



Recent advances in macromolecular X-ray crystallography have laid a solid foundation from which a production pipeline optimized towards the determination of large numbers of structures can be constructed. Industrialization of the process will require the assembly of a linear series of high-throughput steps from target selection to structure interpretation, in which each step must be successfully completed before the next step is attempted (see Box 1 for a basic description of the steps involved in X-ray crystallography). It will also require the automation, miniaturization, parallelization, and optimization of existing crystallographic approaches. Improvements in the processes will have a significant impact on both the efficiency and the effectiveness of experimental techniques in structural biology. These developments are critical for structural genomics efforts as well as for rational drug discovery using high-throughput macromolecular crystallography. When implemented, these techniques should usher in a new era of structure-based biological discovery.

This review focuses on the process steps starting from the crystallization of protein samples to the collection and preliminary analysis of X-ray data. We describe each important task within these steps, identifying possible bottlenecks and efforts to overcome them primarily through automation. Other reviews in this issue focus on steps that occur before (protein production; see the article by Edwards and colleagues) and after (diffraction data analysis; see the article by Lamzin and Perrakis) those discussed here.

High-throughput protein crystallization

The crystallization step normally commences after the successful preparation of highly purified and soluble samples[1]. Attempts are then made to explore the phase space of a protein system by slowly moving the system towards a meta-stable supersaturated state. The actual crystallization conditions can at present not be determined *ab initio*, and the standard protocol is to optimize several solution variables (in a combinatorial approach) known to induce nucleation, such as pH, ionic strength, temperature, and specific concentrations of organic additives, salts and detergent. A complete exploration of this multi-dimensional condition space is impractical and reduced sampling approaches have been developed[2,6]. These methods have been successful, but are limited by the amount of protein available and the thoroughness with which this poorly defined multi-dimensional parameter space can be covered.

Automation of these crystallization processes was a focus at the 8th International Conference on the Crystallization of Biological Macromolecules (ICCBM8)[3]. New generation robots have been developed for the automated production of high-quality protein crystals at the Hauptman-Woodward Medical Research Institute, at the Protein Structure Factory in Germany, and by a collaboration between the Lawrence Berkeley National Laboratory (LBNL) Bioinstrumention Group, the Genomics Institute of the Novartis Research Foundation (GNF), and Syrrx, Inc. (a company devoted to high throughput structure biology)[4,5]. These integrated systems are capable of performing ~10,000 to >100,000 trials a day, using an expanded search of this multi-dimensional condition space[6]. Additionally, both success and failure data can accurately be accumulated for data mining and analysis for future improvements. Both the Hauptman-Woodward and LBNL/GNF/Syrrx system reduce the protein requirements by miniaturization of the experiments to nanoliter volumes. Imaging and analysis of these samples remains a challenge although several advancements have recently demonstrated they can be accomplished in a high throughput manner.

Crystal harvesting and storage for shipping

Once crystals are obtained, they have to be harvested and prepared for data collection. In most cases, structural genomics efforts will rely on synchrotron radiation facilities for efficient and rapid collection of diffraction data (see Box 2 for a basic description of synchrotron radiation). Therefore, samples must be packaged and stored for shipping. One breakthrough in macromolecular crystallography synchrotron data collection has been the routine freezing of protein crystals[7,8]. This is particularly important for multi-wavelength anomalous dispersion (MAD) data collection, where radiation decay can have a serious impact on the experiment. Flash-cooled crystals are stored at cryogenic temperatures and transported to data collection facilities, an approach described by Bob Sweet at Brookhaven National Laboratory as "Protein Crystallography by FedEx™"

[1]Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, USA. [2]Syrrx, Inc., San Diego, California 92121, USA. [3]Stanford Synchrotron Radiation Laboratory, Stanford University, Stanford, California 94305, USA. [4]Berkeley Center for Structural Biology, Physical Biosciences Division, MS6-2100, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA. [5]The Genomics Institute for the Novartis Research Foundation, San Diego, California 92121, USA. Correspondence should be addressed to R.C.S. *email: stevens@scripps.edu*

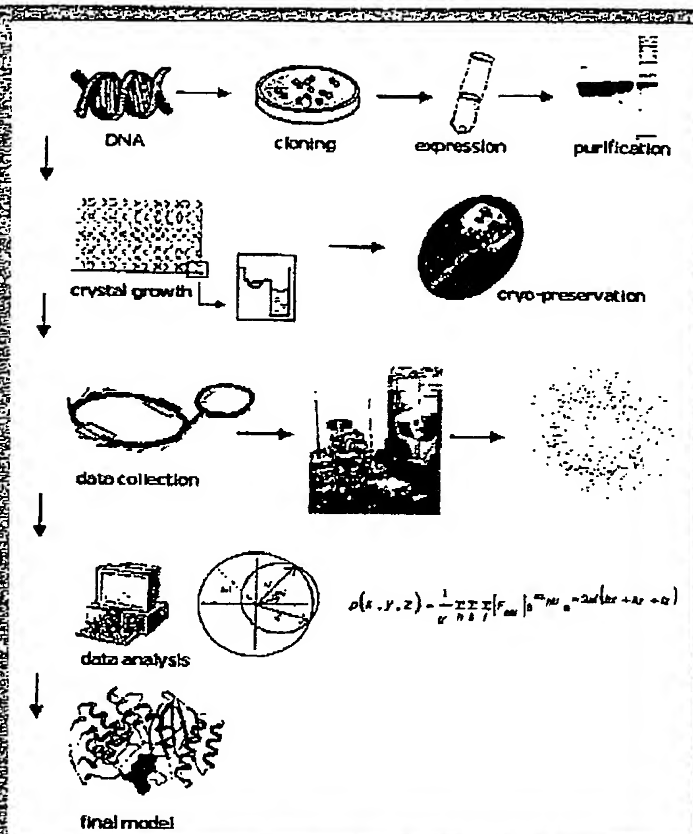
Box 1 Determining protein structures by X-ray crystallography

Protein structure determination involves the following steps: (i) preparation of highly purified protein samples, (ii) crystallization, (iii) measurement of diffraction data, (iv) solving the phase determination problem using natural or artificially incorporated heavier atoms, (v) phase and electron density calculations, (vi) map interpretation and model building and (vii) model refinement.

Incoming X-rays are diffracted by the electrons of the protein atoms to produce diffraction patterns from which a three-dimensional structure of a protein can be derived. In diffraction experiments, one is able to measure the intensity of the scattered beam but not the phases relating these scattered waves. X-ray crystallographic methods developed during the last few decades have focused on improving the measurements and recovering the phase information.

The diffraction experiment is simply the process of orienting a protein crystal in an intense X-ray beam and providing a mechanism for detecting and [illegible] the scattered beams [illegible]. Most modern detectors are multi-element charge coupled device (CCD) systems, which are capable of accurately measuring even weakly scattered X-ray beams. The majority of current diffraction data collection is done using the very intense and tunable X-ray beams from synchrotron storage rings (see Box 2).

Two advances in X-ray crystallographic methodology developed over the past two decades are cryo-freezing of protein crystals, which significantly reduces the radiation damage from the intense X-ray beam, and the development of the multi-wavelength anomalous dispersion (MAD) method. The MAD method relies upon the tuneability of synchrotron radiation to different wavelengths to select for the absorption edge of metal atoms incorporated into protein crystals. This process facilitates the solving of the phase problem. In particular, incorporation of seleno-methionine to substitute for natural methionine residues has tremendously improved the ability to rapidly and routinely determine protein structures.



(ref. 9). Currently, these processes are done manually. Researchers work under a microscope to select a sample. The crystal is mounted into a cryo-loop, flash cooled at liquid nitrogen temperature, and subsequently stored in a shipping dewar. Routine handling of larger numbers of crystals will clearly require automation and resolution of other material handling issues, such as the ability to automatically identify each unique sample as it is mounted in the data collection station.

The task of harvesting crystals from multi-well plates represents a challenging automation problem. A prototype system was designed by Oceaneering Space Systems (Houston, Texas), under contract to the University of Alabama at Birmingham, for use in microgravity environments[10]. The system removes crystals and a small amount of mother liquor from the crystallization experiment. It isolates candidate crystals in a liquid bridge formed between two pipette tips, moves a robotic tip with a cryo-loop through the liquid bridge, and captures and freezes the crystal sample. As the system is designed to work on the space shuttle and international space station, a small robot with six degrees of freedom handles all manipulations. The robot is remotely controlled, allowing earth-based scientists to make decisions regarding crystal harvesting. This represents the first prototype from which subsequent systems can be developed using similar or different approaches.

A workshop, organized by scientists from the Advanced Light Source (ALS), the Advanced Photon Source (APS) and the Stanford Synchrotron Radiation Laboratory (SSRL), was recently held at Stanford University[11] to discuss different approaches to macromolecular crystal sample storage, mounting and characterization. Cryogenic storage, transport and retrieval systems require robotic sample handling at liquid nitrogen temperatures. At the meeting it was proposed that a standardized design will eliminate the need to change storage devices when using beam lines at different synchrotrons. Several projects are currently underway to develop and test prototypes. One example of a conceptual design from SSRL is depicted in Fig. 1, which shows a 96-pin cassette that holds 96 crystals at liquid nitrogen temperatures and the robotic system that extracts individual samples from the

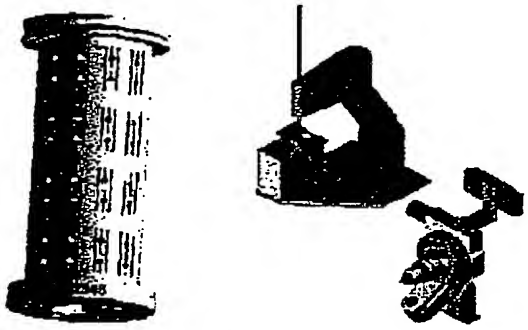


Fig. 1 On the left is the 96-sample cassette, which holds and retains flash-cooled crystals at liquid nitrogen temperatures. On the right is the conceptual design of a transfer robot that removes the flash-cooled sample from the cassette and transfers it at liquid nitrogen temperatures to mount it on the diffractometer. This system is the conceptual design of the SSRL SMB resource funded by the NIH-NCRR and DOE-BER.

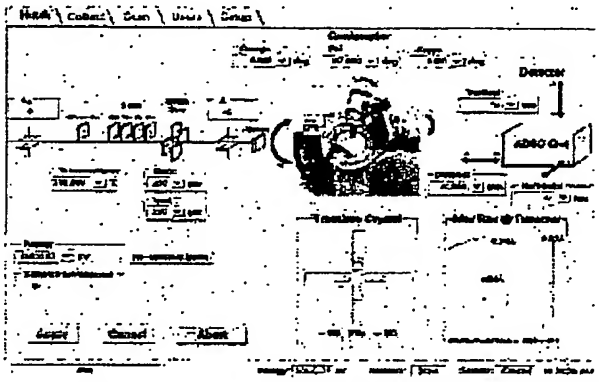


Fig. 2 'Hutch' window of BLU-ICE showing the intuitive interface that allows the experimentor to interact with the synchrotron beam line instrumentation. Complex hardware systems are integrated in the software package to allow ease of access for optimized design of experimental procedures. The photon energy can be changed by modification of a single number, but this change actually involves movement of several optical elements and table positions to new positions.

storage device and mounts them on a diffractometer at the synchrotron beam line. The cassette is bar-coded and each crystal location will be identifiable by a coordinate system, allowing for the unique identification of each sample.

Data collection at synchrotron beam lines

Synchrotron beam lines were initially developed as experimental stations, not for high throughput data collection. Therefore, data collection experiments currently require the user to enter the beam line hutch, mount and center a crystal, and exit/lock the hutch for every individual sample. These tasks result in a tremendous loss of valuable and scarce synchrotron beam time. Table 1 outlines estimates made by the Southeast Regional Collaborative Access Team (SER-CAT) group at APS of the amount of time used for a manual data collection procedure *versus* a potential automated procedure. In reality, no experimenter can keep up the optimal manual pace outlined in the table over a 24 hour period. Therefore, the throughput gain due to automation can be even more significant.

Automated approaches to data collection have become possible in recent years with the availability of reliable synchrotron sources, stable X-ray optics, rapid readout charge coupled device (CCD) detectors and high-precision diffractometers. Many of the synchrotron beam line development groups are currently implementing various automation systems that allow for mail-in crystallography and the development of research collaboratories to solve challenging problems in structural biology[11]. Advanced instrumentation and sophisticated software environments will enable full automation of synchrotron beam lines for high-throughput data collection to meet the goals of the integrated structure determination pipeline. These systems will allow the overwhelming number of crystallographic projects to be successfully pursued by many research groups. The data acquisition systems currently implemented by the National Synchrotron Light Source (NSLS) and SSRL are capable of remote operation and joint execution of experiments[12]. Fig. 2 shows the setup window of the system called Beam Line Unification in an Integrated Control Environment (BLU-ICE)[13], which is an example of a completely integrated software environment that functions as a distributed control system for crystallo-

Table 1 Task analysis showing typical time needed for data collection and estimated time savings with automation[1]

	Current manual estimate (seconds)	Automated estimate (seconds)
Open the hutch door and walk to the goniostat	25	0
Move the detector back	10	10
Retrieve the old sample	20	5
Mount a new sample	20	5
Align the new sample	90	45
Move of the detector back to data collection position	10	10
Set-up the interlock, leave the hutch and close the door	30	0
Wait for the end of the warning announcement	30	0
Open the photon shutter and return to the console	5	0
Beam stop alignment check	120	0
Start exposure	10	10
Total mounting time for each crystal	370	85
Repeat this for 3 crystals (needed to find a good crystal)	1,110	255
Data collection time	600	600
Total time for a data set	1,710	855

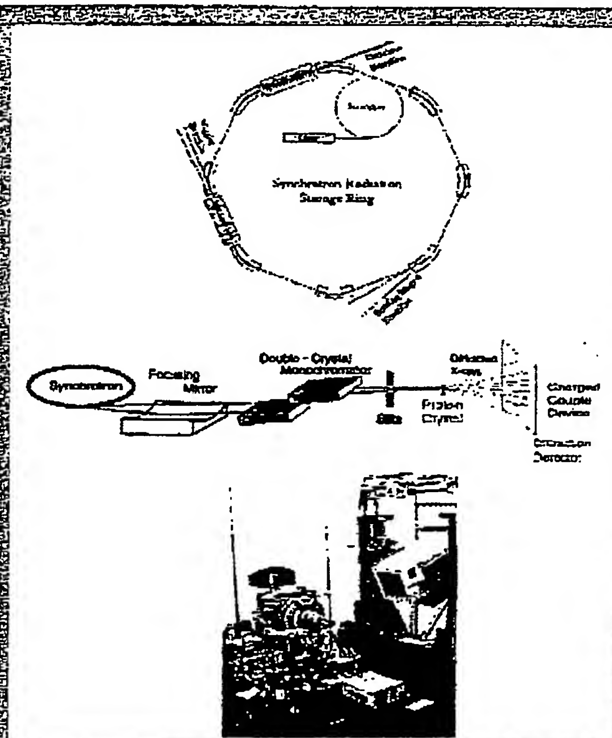
[1]Data collection times vary grossly and are crystal-specific. In reality, no experienced experimenter can keep up the optimal manual pace outlined in the table over 24 hours, nor are all users experienced at the same level. Table was kindly provided by B.C. Wang, principal investigator of the SER-CAT consortium at APS.

Box 2 Using synchrotron radiation for structural genomics

Synchrotron radiation is now indispensable for structural studies and will be the primary X-ray source for structural genomics efforts. Biologists started using synchrotron facilities in the early 1970s and by the 1980s synchrotron radiation was helping to push the envelope by allowing crystallographers to work with increasingly larger molecules such as virus particles and the intact ribosome [illegible]

Synchrotron radiation is produced when a high energy particle beam changes direction in magnetic devices such as bending magnets, undulators or wigglers. The particle beams are either electron or positron beams that are generated by a linear accelerator (LINAC) [illegible]

[illegible]



[illegible] A schematic of a generic synchrotron source and a beamline (top) and a picture of a standard experimental hutch (bottom) are shown. A typical synchrotron radiation source consists of a linear accelerator (LINAC), a booster ring, a storage ring and the beamline. The sources of radiation are the magnetic devices that guide the electron path. These devices are typically bending magnets, wigglers and undulators. Bending magnets are the arcs of the polygon defining the synchrotron storage ring. Wigglers and undulators are installed in the straight sections; these devices consist of a series of alternating magnets causing the electron beam to wiggle, resulting in the emission of very intense X-ray beams with different spectral characteristics. The X-rays are guided and conditioned by using optical devices such as focusing mirrors and monochromators to project a focused, well-collimated and monochromatic beam on the sample, which is mounted on a diffractometer (middle). Finally, the experimental hutch contains the final beam conditioning system, sample mounting devices and diffraction detector system.

graphic data collection. With BLU-ICE, users anywhere in the world can observe or have full control of all experimental instrumentation at a synchrotron hutch through a single, intuitive graphical interface. This control environment has eliminated the requirement for the experimenter to operate a beam line using multiple computer interfaces and/or mechanical devices. This unified access to all beam line instrumentation provides a clear path for further automation of other process steps.

Automated crystal mounting, viewing, and centering

Projects are currently underway to implement beam line automation leading to the time savings outlined in Table 1. Technical and process issues are being addressed to automate the mounting, centering and characterizations of crystals. During the Stanford workshop[11], Steve Muchmore from Abbott laboratories described an operational system that allows their group to collect multiple data sets, uninterrupted, on a standard laboratory rotating anode based system. What made this possible was their use of an automated sample changer and alignment system. The system consists of a three-axis manipulation robot fitted with a custom designed tool for crystal mounting, and a video camera used to obtain images of mounted crystals to perform the automated alignment procedures. These components have been integrated with a commercially available CCD detector system. The system includes a sample storage rack containing up to 63 pre-frozen crystals mounted on cryoloops. The racks are stored in an open dewar in which a layer of liquid nitrogen gas is used to reduce icing. Although constructed for use with a non-synchrotron X-ray source, the design and implementation allow for integration into synchrotron beam lines.

A second system is currently being developed specifically for synchrotron beam line use. The bioinstrumentation group at LBNL, in collaboration with ALS and researchers at GNF as well as Syrrx are developing a first generation automated crystal

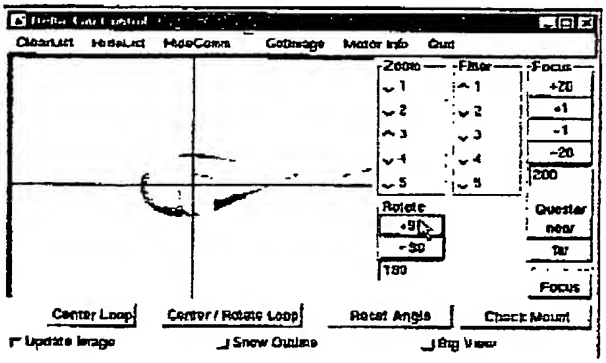


Fig. 3 Autocentering of protein crystals at the Advanced Light Source. By clicking on the crystal location, the sample is automatically centered in the X-ray beam.

recognition and alignment system designed to work at synchrotron beam lines. The system employs a task-specific design in which a standard cryovial is moved to a goniometer. An electromagnet holds the magnetic cap and sample while the vial is replaced into a liquid nitrogen dewar. Automation of crystal alignment using an intelligent software system is currently under development (Fig. 3). When operational, this system will allow for the screening and data collection of several hundred crystals a day.

Finally, the handling of very small crystals is being made possible by the development of newly designed microcrystal diffractometers such as the Grenoble EMBL/ESRF microfocus beam line ID13 (refs. 14,15). The ID13 beamline is designed to allow maximum precision positioning combined with ease of usage necessary for handling very small crystals (down to 5 μm). The design centers around a parallax-free video microscope used to view both the sample and a beam localization scintillator. A motorized sample centering system, ϕ-axis positioning tables, beam definition aperture for beam sizes of 10–200 μm, and beamstop allow for computer-aided automation. Currently, sample loading is done manually and crystal alignment is performed *via* a graphical user interface. Under development is an automated sample changer for pre-frozen crystals capable of handling up to 24 samples stored in cylindrical racks. Each of these samples can be transferred automatically to/from the goniometer axis. Use of the micro-diffractometer allows for the use of very small crystals and also allows for diffraction from localized areas of twinned crystals. These features are desirable for high-throughput studies, especially for those using reduced protein volumes in crystallization experiments that are expected to produce smaller crystals.

Diffraction quality analysis, indexing, and data collection

Most structure determinations require the screening of multiple crystals in order to select the most suitable sample with regard to diffraction quality and orientation. The number of crystals used depends upon the complexity of the project; for example, very few test crystals are required for determination of additional structures, such as of a mutant protein or a protein in complex with an inhibitor, but hundreds are required for more difficult membrane proteins or multi-domain complexes. The mounting/unmounting and initial crystal characterization represent time consuming steps, which can be made more efficient by automation. Robotic handling and data maintenance reduce the chance of human error, which can be introduced in manual methods by the required repeated handling of each sample, as well as the data assessment of many different samples. Crystallographic software development groups are currently developing and testing software to evaluate diffraction quality and establish optimal data collection strategies using currently available beam line capabilities (such as multi-circle diffractometers, high-speed CCD detectors, and detector off-set).

Conclusion

Industrialization of the structure determination process will increase final throughput numbers and lead to an enhanced success rate and higher quality of derived three-dimensional models. It will result in the establishment and population of databases that contain accurate information on all process steps, including both successes and failures. These databases will contain information related to target selection, protein expression/crystallization, data collection and refined structures. Systematic analysis of these databases will display trends and provide answers to questions for rational experimental design so that there will ultimately be less reliance on anecdotal stories that historically have directed experiments on an almost trial and error basis.

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Associations with structural genomics

E.A. is a consultant for Syrrx. P.K. is a member of the Joint Center for Structural Genomics (JCSG) between TSRI, UCSD, and Stanford. T.E. is Head of the Macromolecular Crystallography Facility at the Advanced Light Source. R.S. is a member of the JCSG and a consultant for the Genomics Institute for the Novartis Research Foundation (GNF). R.S. is also a founding scientist of Syrrx, a company focused on high throughput protein crystallography for drug discovery.

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